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ORIGINAL ARTICLE

# Detection of *Pseudomonas aeruginosa* isolates carrying the $bla_{OXA-142}$ extended-spectrum β-lactamase gene in Taiwan

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**KEYWORDS**
Extended-spectrum β-lactamase;
Integron;
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*Pseudomonas aeruginosa*

*Background/Purpose:* The emergence of extended-spectrum β-lactamase (ESBL) OXA-142 gene ($bla_{OXA-142}$) in *Pseudomonas aeruginosa* has been reported in Bulgaria and other European countries, but rarely in Asia.
*Methods:* From January 2009 to December 2012, 90 *P. aeruginosa* isolates with reduced susceptibility or resistance to ceftazidime (minimum inhibitory concentration $\geq$ 8 mg/L) were screened for ESBL and other broad-spectrum β-lactamase genes by polymerase chain reaction and sequencing. Clonal relationship of the isolates was determined by pulsed-field gel electrophoresis.
*Results:* Three isolates were positive for ESBL production, exhibited resistance to ceftazidime, and carried the $bla_{OXA-142}$ gene. The $bla_{OXA-142}$ gene was integrated into class 1 integron. Using Southern blot analysis, $bla_{OXA-142}$-containing integron was found to be located on a plasmid in

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all three isolates. Eleven strains of *P. aeruginosa* carrying $bla_{OXA-17}$ gene were found. The *oprD* mutation was found in all the 21 ESBL strains of *P. aeruginosa*.
*Conclusions:* This study confirmed the presence of $bla_{OXA-142}$-positive *P. aeruginosa* isolates in Taiwan.


## Introduction

*Pseudomonas aeruginosa* is one of the most common causes of healthcare-associated infections.[1,2] The mechanism of resistance to β-lactam drugs of *P. aeruginosa* can be attributed to the acquisition of the β-lactamase gene, inactivation of *oprD* causing outer-membrane impermeability, or expression of broadly specific multidrug efflux pump systems.[3–5] The main β-lactamase genes include those encoding for carbapenemase, extended-spectrum β-lactamase (ESBL), and AmpC β-lactamase; all of which may integrate into mobile elements that can carry multiple resistance determinants and enable *P. aeruginosa* to acquire multiple drug resistance. Recently, multidrug-resistant *P. aeruginosa* isolates carrying ESBL $bla_{OXA-142}$ gene have emerged in Bulgaria.[6] The ESBL $bla_{OXA-142}$ gene is an Amber class D β-lactamase gene. The OXA-type β-lactamases, which have large sequence variation between individual enzymes, can be categorized into five groups. The β-lactamases OXA-10 and OXA-17 belong to OXA Group I. The enzyme OXA-142 is an extended-spectrum derivative of OXA-10-related enzymes. According to previous studies, including the research project from the Centers for Disease Control in Taiwan, no $bla_{OXA-142}$-positive *P. aeruginosa* isolates have been previously reported.[7,8] Epidemiological surveys have shown that *P. aeruginosa* sequence type (ST) 235 is predominantly responsible for the dissemination of *P. aeruginosa* isolates in Europe, Asia—Pacific area, and South America.[9–14] Due to the multidrug-resistance nature of *P. aeruginosa*, clinical treatment is often difficult, where *P. aeruginosa* continues to be frequently involved in many healthcare-associated infections.

The aim of this study was to detect ESBL gene, especially $bla_{OXA-142}$ and other carbapenemase-encoding genes among ceftazidime-resistant *P. aeruginosa* isolates in a medical center in Taiwan. The clonal relationship of the isolates, plasmid localization of the $bla_{OXA-142}$ gene, and clinical features of patients harboring isolates carrying $bla_{OXA-142}$ were also determined. For further investigation of carbapenems resistance, we also described genetic events that could lead to the inactivation of *oprD*, causing outer-membrane impermeability of *P. aeruginosa*.

## Methods

### Bacterial isolates collection, antimicrobial susceptibility testing and patients characteristics

The 892 *P. aeruginosa* isolates were collected from January 2009 to December 2012 in Mackay Memorial Hospital, a 2200-bed position hospital, in Taiwan. The 10.09% (90/892) *P. aeruginosa* isolates with reduced susceptibility to ceftazidime [minimum inhibitory concentration (MIC) $\geq$ 8 mg/L] were collected in the initial collection. Identification was performed using the VITEK 2 system (bioMérieux Vitek Systems, Hazelwood, MO, USA). MICs were determined via agar dilution.[15] The MICs of tested antibiotics, which included ceftazidime, ciprofloxacin, amikacin, gentamicin, and carbapenems (imipenem, meropenem and doripenem) were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[16] The MICs of colistin were interpreted according to the CLSI and European Committee on Antimicrobial Susceptibility Testing breakpoint.[17] Finally, a retrospective review of the patient medical charts was performed to analyze the clinical information in patients harboring *P. aeruginosa* positive for $bla_{OXA-142}$. This study was approved by the Mackay Memorial Institutional Review Board with the protocol number 13MMHIS218.

### Carba NP test

Production of carbapenemases was phenotypically tested with the Carba NP test. The supernatant from the enzymatic bacterial suspension of the tested strain was mixed with 100-μL aliquots of a 1-mL solution that was made from 3 mg imipenem monohydrate (USP, Rockville, MD, USA), phenol red (Merck Millipore, Billerica, MA, USA) solution, and 0.1 mmol/L $ZnSO_4$ (Merck Millipore) at pH of 7.8. A mixture of the phenol red solution and the testing enzymatic suspension was incubated at 37°C for a maximum of 2 hours.[18]

### Polymerase chain reaction and sequencing

The 90 *P. aeruginosa* isolates were screened for carbapenemase genes such as $bla_{IMP}$, $bla_{VIM}$, $bla_{NDM}$, $bla_{SPM}$, $bla_{AIM}$, $bla_{DIM}$, $bla_{GIM}$, $bla_{SIM}$, $bla_{KPC}$, $bla_{BIC}$, and $bla_{OXA-48}$. They were also screened for ESBL genes that included $bla_{VEB}$, $bla_{PER}$, $bla_{GES}$, $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX}$, $bla_{BEL}$ and $bla_{PSE}$ from Class A; and $bla_{OXA\text{-}group\ I}$, $bla_{OXA\text{-}group\ II}$, $bla_{OXA\text{-}group\ III}$ from Class D (Table 1).[19–22] The screening for Class 1 integron and sequencing of its cassettes was performed (Table 1).[20,21] The detection of *oprD* genes and subsequent sequencing procedures were also performed (Table 1).[22] The polymerase chain reaction (PCR) preparation procedure was as follows: bacteria were boiled in sterile water for 10 minutes; the supernatant was collected and used as DNA sources for PCR; the 25-μL reaction mixture consisted of 1× S-T Gold buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 20 pmol of each

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**Table 1** Major oligonucleotide primers used in polymerase chain reaction

| Primer | Sequence (5′—3′) | Use | Refs |
|---|---|---|---|
| ABD1 | TAT CGC GTG TCT TTC GAG TA | $bla_{OXA}$ detection & probe for Southern blotting | 19 |
| ABD4 | TTA GCC ACC AAT GAT GCC C | $bla_{OXA}$ detection & probe for Southern blotting | 19 |
| pse-1-1a | GTG TGA CAA TCA AAA TTA TG | $bla_{PSE-1}$ detection | 20 |
| Int1-6A | ATA AGC CTG TTC GGT TCG TA | Integron determination | 21 |
| qacE-2B | GAT TTT AAT GCG GAT GTT GCG | Integron determination | 21 |
| 379OXA-10F | GTT GGC GAA GTA AGA ATG CAG | Integron sequencing | This study |
| 399OXA-10R | CTG CAT TCT TAC TTC GCC AAC | Integron sequencing | This study |
| 3end OXA-1a | GAC AAC GAA AGT AAG TTG CC | Integron sequencing | This study |
| 59OXA | CCA GCT AAT GCC GTA CTC G | Integron sequencing | This study |
| 5end OXA-1a | CCT AGG AAC AAG GCT TGT CC | Integron sequencing | This study |
| 3end3568OXA-2a | CGA AGC GCC GCG CCC AGT GG | Integron sequencing | This study |

pse-1-1b AGC GCG ACT GTG ATG TAT AA $bla_{PSE-1}$ detection[21].
OprD.F GGA ACC TCA ACT ATC GCC AAG *oprD* detection[22].
OprD.R GTT GCC TGT CGG TCG ATT AC *oprD* detection[22].

primer. The PCR amplicons were purified using ExoSAP-IT reagent (USB, Cleveland, OH, USA), and both strands were sequenced using the standard dideoxynucleotide method in an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequence similarity searches were performed with the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## Pulsed-field gel electrophoresis and multilocus sequence typing

The 90 *P. aeruginosa* isolates with reduced susceptibility (MICs of ceftazidime $\geq$ 8 mg/L) were typed by pulsed-field gel electrophoresis (PFGE) following digestion of intact genomic DNA with *Spe*I (Biolabs, Beverly, MA, USA). The DNA fragments were separated on 1% (w/v) SeaKem GTG agarose gels in 0.5% Tris—borate—EDTA buffer, in a CHEF Mapper apparatus (Bio-Rad, Hercules, CA, USA) with a potential of 6 V/cm, pulsed from 5 seconds to 35 seconds, for 22 hours at 14°C.[23] The completed gels were stained with ethidium bromide and photographed with UV light. The *Spe*I restriction profiles were initially compared with each other by visual inspection and isolates were considered to be closely related if they showed differences of less than three bands.[24] Computer-assisted analysis was also performed using BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was performed by the unweighted pair group method with mathematical averaging, and DNA relatedness was calculated using the band-based Dice coefficient with a tolerance setting of 1.0% band tolerance and 1.0% optimization setting for the whole profile.[25] Isolates were considered to belong to the same cluster when similarity coefficient reaches 80%.[26] Finally, multilocus sequence typing (MLST) was performed for the $bla_{OXA}$-positive *P. aeruginosa* isolate in accordance with the protocol available at http://pubmlst.org/paeruginosa/.

## Plasmid localization of the $bla_{OXA-142}$ gene

To assess the plasmid location of $bla_{OXA-142}$, Southern blot analysis was performed with plasmid DNA. Plasmid DNA was extracted by using a plasmid maxi kit (Qiagen, Valencia, CA, USA) and underwent agarose gel electrophoresis. After transferring the DNA onto a nylon membrane (PerkinElmer, Boston, MA, USA), the DNA was UV crosslinked and hybridized with the PCR-generated probes specific for $bla_{OXA-142}$. Labeling of the probes and hybridization signal detection were carried out with digoxigenin DIG DNA Labeling and Detection kit (Roche Diagnostics, Basel, Switzerland).

# Results

## Molecular typing of *P. aeruginosa* isolates positive for $bla_{OXA}$ and carbapenamase genes

Twenty-one (21/90, 23.33%) *P. aeruginosa* isolates were positive for ESBL genes, including four for $bla_{OXA-10}$, 11 for $bla_{OXA-17}$, three for $bla_{OXA-142}$, and three for $bla_{PSE-1}$. The number of $bla_{OXA}$-positive *P. aeruginosa* isolates from 2009 to 2012 is shown in Fig. 1. Seven (7/90, 7.78%) *P. aeruginosa* isolates were positive for carbapenemase genes, including six with $bla_{VIM-2}$ and one with $bla_{VIM-3}$.

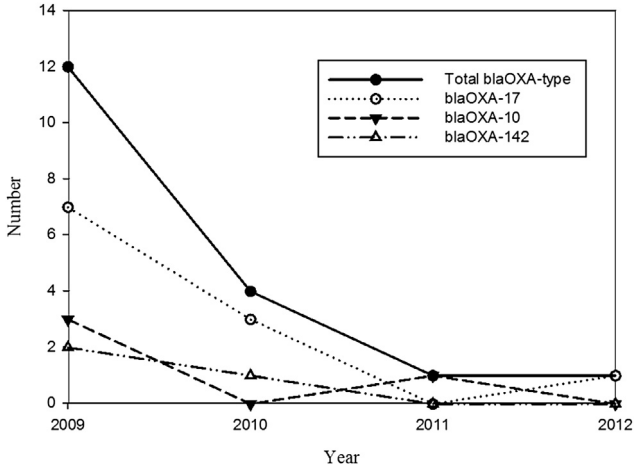


**Figure 1.** Number of $bla_{OXA-type}$ *Pseudomonas aeruginosa* isolates from 2009 to 2012.

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Using PCR methods, these ESBL-positive isolates were negative for $bla_{IMP}$, $bla_{VIM}$, $bla_{NDM}$, $bla_{SPM}$, $bla_{AIM}$, $bla_{DIM}$, $bla_{GIM}$, $bla_{SIM}$, $bla_{KPC}$, $bla_{BIC}$, and $bla_{OXA-48}$ carbapenemases genes. All three isolates with $bla_{OXA-142}$ belonged to ST 235 by MLST and resulted in closely related patterns with PFGE. Comparison of PFGE patterns among ESBL-positive isolates is demonstrated in Fig. 2. These 90 *P. aeruginosa* isolates were classified by 46 PFGE patterns. Four isolates of $bla_{OXA-10}$, three isolates of $bla_{OXA-142}$, one of 11 isolates of $bla_{OXA-17}$, one of 11 isolates of $bla_{OXA-17}$, and the other nine of 11 isolates *P. aeruginosa* were classified as PFGE type 19, PFGE type 37, PFGE type 35, PFGE type 36, and PFGE type 37, respectively. The $bla_{OXA-142}$ gene was integrated into Class 1 integron. Southern blot analysis showed that $bla_{OXA-142}$ was located on the plasmid in all three isolates with the $bla_{OXA-142}$ β-lactamase gene.

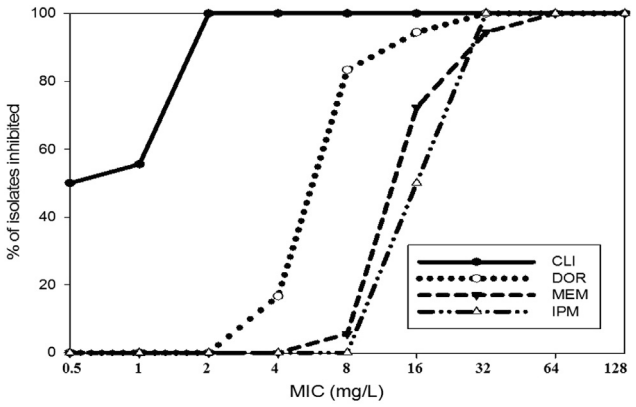


**Figure 3.** The MICs of *P. aeruginosa* isolates carrying $bla_{OXA}$ genes for colistin (CLI) and three carbapenems (DOR: doripenem; MEM: meropenem; IPM: imipenem).

## Susceptibility testing of *P. aeruginosa* isolates carrying $bla_{OXA-142}$

Among the *P. aeruginosa* isolates carrying $bla_{OXA-142}$ PA04, PA17 and PA55, the MICs of imipenem were 16 mg/mL, 32 mg/mL and 16 mg/L, respectively. The MICs of meropenem were 16 mg/L for all three isolates. The MICs of doripenem were 4 mg/mL, 8 mg/mL and 8 mg/L, respectively. When compared with the other carbapenems, doripenem showed the lowest MICs in these three ceftazidime-resistant *P. aeruginosa* isolates. The MICs of colistin in these three isolates were 2 mg/L (Fig. 3). By using the Carba NP test, these three isolates all tested negative for carbapenemase. The MICs of *P. aeruginosa* isolates carrying $bla_{OXA}$ genes for colistin and three carbapenems were demonstrated in Fig. 3. The MICs of other tested antibiotics, including ciprofloxacin, amikacin and gentamicin, were higher than the cut-off points of resistance.

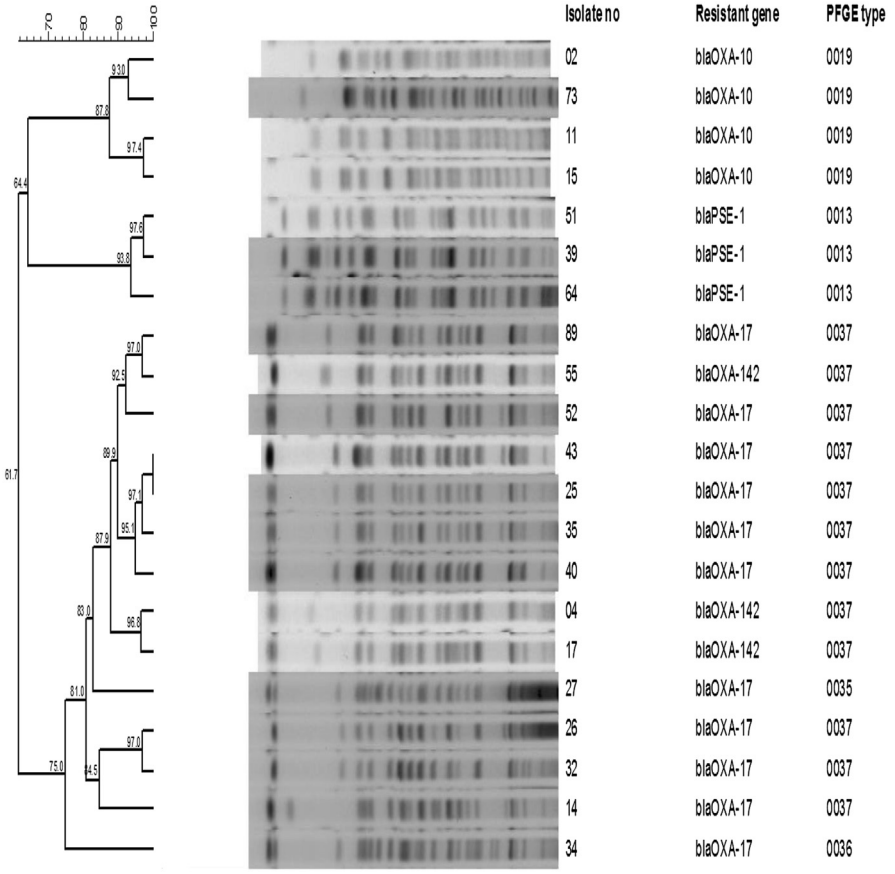


**Figure 2.** Pulse-field gel electrophoresis of *Pseudomonas aeruginosa* isolates positive for extended-spectrum β-lactamase.

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### Characteristics of patients infected with *P. aeruginosa* isolates carrying $bla_{OXA}$

Characteristics of patients infected with *P. aeruginosa* isolates carrying $bla_{OXA}$ are shown in Table 2. Among the patients infected with $bla_{OXA-142}$ positive *P. aeruginosa*, Patient 7 (PA04) had diabetes and was bed-ridden due to a cerebrovascular accident; he also had aspiration pneumonia complicated with bacteremia. The patient was treated with colistin after blood culture results were available, but he died within 24 hours after treatment was initiated. Patient 5 (PA17) had asymptomatic urinary tract infection associated with the use of a urinary catheter. The urinary catheter was subsequently removed and the patient was discharged without complications. Patient 6 (PA55) underwent drainage of a retroperitoneal abscess that was followed by secondary wound infection with $bla_{OXA-142}$-positive *P. aeruginosa*.

### Detection of *oprD* mutation

None of the isolates carried carbapenemases genes, therefore, we determined whether there was a mutation in the *oprD*. An *oprD* mutation was found in all of the ESBL-positive *P. aeruginosa*. In the $bla_{OXA-10}$-positive *P. aeruginosa*, the *oprD* mutation resulted from a frameshift mutation. Frameshift mutations were found in the PA4 and PA17 isolates of $bla_{OXA-142}$-positive *P. aeruginosa*. The presence of a premature stop codon was also found in the Pa55 isolate of $bla_{OXA-142}$-positive *P. aeruginosa*. In $bla_{OXA-17}$-positive *P. aeruginosa*, *oprD* mutation was shown to arise from various types of frameshift mutations and premature stop codons. The *oprD* mutation of OXA type ESBL-positive *P. aeruginosa* is summarized in Table 2. In the $bla_{PSE-1}$-positive *P. aeruginosa*, *oprD* mutation resulted from both frameshift mutation and premature stop codon. PAO1 was used as a reference for wild-type *P. aeruginosa*.

## Discussion

This study confirmed the presence of $bla_{OXA-142}$-positive *P. aeruginosa* in Taiwan. The $bla_{OXA-142}$-positive *P. aeruginosa* isolates first emerged in Europe, and we have now also detected the presence of $bla_{OXA-142}$-positive *P. aeruginosa* isolates in Taiwan. For the purpose of epidemiological surveys, we determined the clonal relationships among the different strains of *P. aeruginosa*. Several molecular typing methods were also used in determining the relationship between *P. aeruginosa* and healthcare-associated infections. The most recognized discriminatory method for molecular typing, PFGE, has been a clinically valuable method in evaluating short-term epidemiological *P. aeruginosa* infections. However, another method, MLST is more appropriate for evaluating long-term evolutionary relationships in *P. aeruginosa* populations. The *P. aeruginosa* ST 235 has been responsible for infections throughout Europe, Asia, and South America.[27]

In this study, the three $bla_{OXA-142}$-positive *P. aeruginosa* isolates were shown to have closely related PFGE patterns, and MLST showed that they belonged to ST 235. The three isolates tested negative for carbapenemase in the Carba NP and PCR screenings. Doripenem exhibited lower MICs to $bla_{OXA-142}$-positive *P. aeruginosa* isolates when compared to the other two tested carbapenems, however, it did not

**Table 2** Characteristics of *Pseudomonas aeruginosa* isolates and patients infected/colonized with isolates carrying $bla_{OXA}$ genes

| No. | PA isolate | MICs (mg/L) | | | | | Gene | *oprD* mutation[a] | Age/sex | ICU stay | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CAZ | IPM | MEM | DOR | CLI | | | | | |
| 1 | PA15 | 64 | 32 | 32 | 32 | 0.5 | $bla_{OXA-10}$ | A | 84/M | No | Survived |
| 2 | PA73 | 64 | 32 | 16 | 16 | 0.5 | $bla_{OXA-10}$ | A | 78/M | No | Survived |
| 3 | PA02 | 64 | 32 | 16 | 8 | 2 | $bla_{OXA-10}$ | A | 52/F | No | Survived |
| 4 | PA11 | 64 | 32 | 8 | 8 | 0.5 | $bla_{OXA-10}$ | A | 76/M | No | Survived |
| 5 | PA17 | 128 | 32 | 16 | 8 | 2 | $bla_{OXA-142}$ | A | 87/M | Yes | Survived |
| 6 | PA55 | 128 | 16 | 16 | 8 | 2 | $bla_{OXA-142}$ | B | 88/F | Yes | Survived |
| 7 | PA04 | 64 | 16 | 16 | 4 | 2 | $bla_{OXA-142}$ | A | 67/F | Yes | Died |
| 8 | PA43 | 32 | 32 | 64 | 16 | 0.5 | $bla_{OXA-17}$ | B | 48/M | Yes | Died |
| 9 | PA34 | 16 | 16 | 32 | 8 | 0.5 | $bla_{OXA-17}$ | A, B | 101/M | No | Survived |
| 10 | PA26 | 16 | 16 | 32 | 8 | 0.5 | $bla_{OXA-17}$ | B | 70/M | No | Survived |
| 11 | PA40 | 16 | 32 | 32 | 8 | 0.5 | $bla_{OXA-17}$ | B | 94/M | No | Survived |
| 12 | PA14 | 16 | 16 | 16 | 8 | 0.5 | $bla_{OXA-17}$ | A, B | 54/M | No | Survived |
| 13 | PA25 | 32 | 16 | 16 | 8 | 2 | $bla_{OXA-17}$ | A, B | 83/M | No | Survived |
| 14 | PA89 | 16 | 16 | 16 | 8 | 2 | $bla_{OXA-17}$ | B | 76/F | Yes | Died |
| 15 | PA52 | 16 | 16 | 16 | 8 | 2 | $bla_{OXA-17}$ | B | 68/F | Yes | Survived |
| 16 | PA32 | 32 | 32 | 16 | 8 | 2 | $bla_{OXA-17}$ | B | 76/F | No | Survived |
| 17 | PA27 | 16 | 32 | 16 | 4 | 1 | $bla_{OXA-17}$ | A, B | 65/F | No | Survived |
| 18 | PA35 | 64 | 16 | 16 | 4 | 0.5 | $bla_{OXA-17}$ | B | 71/F | Yes | Died |

[a] *oprD* mutations A and B resulted in frameshift mutation and premature stop codon, respectively.
CAZ = ceftazidime; CLI = colistin; DOR = doripenem; ICU = intensive care unit; IPM = imipenem; MEM = meropenem; MIC = minimum inhibitory concentration.

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show sensitivity to the three isolates using either the EUCAST or CLSI criteria. In contrast, colistin showed activities against all three *bla*$_{OXA-142}$-positive *P. aeruginosa* isolates using either the EUCAST or CLSI criteria.[16,17]

Two of the three isolates in our study caused healthcare-associated infections and one isolate was regarded as a noninfectious colonizer. One patient died from *bla*$_{OXA-142}$-positive *P. aeruginosa* bacteremia soon after the blood culture result become available, despite treatment with colistin. Due to concerns of potential renal toxicity of colistin, physicians seldom prescribe colistin when empirically treating possible Gram-negative bacterial infections. This inadequate medication may lead to acute life-threatening situations when the infections are caused by *bla*$_{OXA-142}$-positive *P. aeruginosa* isolates. All of the *bla*$_{OXA-142}$ harboring integrons were integrated into plasmids. The *oprD* mutation was also found in all of the ESBL strains of *P. aeruginosa*. Frameshift mutation was found in PA04 and PA17 of *bla*$_{OXA-142}$-positive *P. aeruginosa* isolates. Premature stop codons were found in PA55 *bla*$_{OXA-142}$-positive *P. aeruginosa* isolate. Previous studies have indicated that the *oprD* mutation plays an important role in resistance to carbapenems.[5,28] Although colistin is sensitive to *P. aeruginosa* isolates carrying *bla*$_{OXA}$ genes *in vitro*, it would be clinically limited in patients with both active infections and renal insufficiency. The multiple resistance mechanism of *P. aeruginosa* isolates carrying *bla*$_{OXA}$ genes lessens the therapeutic options.

In conclusion, we report the detection of the first *bla*$_{OXA-142}$-positive *P. aeruginosa* isolates from Taiwan. The ESBL *bla*$_{OXA-142}$ gene was integrated to Class 1 integron in all three isolates. All three isolates had *bla*$_{OXA-142}$-containing integron in plasmids. The commonest strain causing *P. aeruginosa* infection in 2009 was *bla*$_{OXA-17}$-positive *P. aeruginosa*. The *oprD* mutation was found in all the ESBL strains of *P. aeruginosa*. Intensive care unit patients who encounter *bla*$_{OXA-142}$-positive *P. aeruginosa* and *bla*$_{OXA-17}$-positive *P. aeruginosa* may develop acute life-threatening infections resulting in mortality.

## Conflicts of interest

All authors declare that they have no competing interests.

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